# Redox Signaling Between Endothelium and Aortic Smooth Muscle: Effect of Dietary Salt

## Supplemental Information

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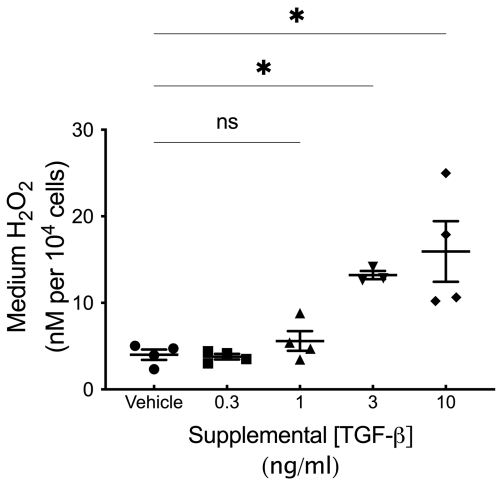
## S.1. *Endothelial cells in culture produce hydrogen peroxide when incubated with TGF-β1.*




**Supplemental Figure 1**. **Endothelial cells produce $H_2O_2$.** Endothelial cells incubated overnight in medium that contained vehicle (4 μM HCl) or active TGF-β in concentrations between 0.3 and 10 ng/ml demonstrated a dose-dependent increase in medium $H_2O_2$ concentrations detected using Amplex® Red (n=3-4 experiments in each group; **P*<0.05; ns, not significant; one-way ANOVA).

## *S.2. Development and characterization of mice lacking Nox4 in endothelium.*

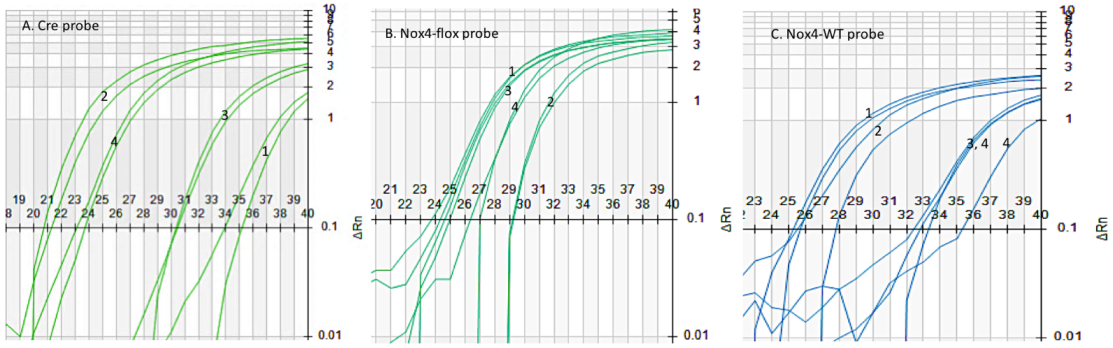
Prior detailed characterization of transgenic mice with the *VE-Cad-CreER$^{T2}$* genotype using the ROSA26R reporter mouse demonstrated widespread recombination in the endothelium of embryonic, neonatal and adult tissues, but not in cells of hematopoietic lineage [1]. Using transgenic mice expressing VE-cadherin-Cre recombinase [2], we generated endothelium-specific *Nox4* knockout mice (*VE-Cad-Cre$^{+}$/Nox4$^{fl/fl}$* genotype); the *Nox4* floxed mouse was a gift from Dr. Junichi Sadoshima [3]. This murine strain (*VE-Cad-Cre$^{+}$/Nox4$^{fl/fl}$*), as well as littermate controls (*VE-Cad-Cre$^{-}$/Nox4$^{fl/fl}$*), were generated using a breeding strategy that only crossed female floxed mice with male Cre mice to minimize the potential genetic (mitochondria) instability and ensure reproducibility. Male VE-Cadherin-Cre mice (*VE-Cad-Cre$^{+}$* genotype) were mated with female mice (*Nox4$^{fl/fl}$* genotype), and then *VE-Cad-Cre$^{+}$* male mice generated from $F_1$ (*VE-Cad-Cre/Nox4$^{flox/wt}$* genotype) were backcrossed to female mice (*Nox4$^{fl/fl}$* genotype). All animals had C57BL/6 genetic background. We confirmed the genotypes of all the mice by PCR genotyping of tail snips. $F_2$ mice were genotyped using Real-Time PCR based on Taqman technology. Three sets of primers and probes were designed to identify *VE-Cadherin-Cre*, *Nox4*-flox and *Nox4*-wt alleles (**Supplemental Table 1**).

**Supplemental Table 1. Primers and probes used to identify the alleles of interest.**

| Genotype | Primer | Probe |
|---|---|---|
| VE-cadherin-Cre | TTAATCCATATTGGCAGAACGAAAACG | CTTAATCATCTAGGAGGAATTC |
| | CAGGCTAAGTGCCTTCTCTACA | |
| *Nox4*-flox | TGGTAAGTATGGCAAGTTCCATTTTCT | CTTAATCATCTAGGAGGAATTC |
| | TCAGACCTGAAGTTCCTATACTTTCTAGAG | |
| *Nox4*-wt | TGGTAAGTATGGCAAGTTCCATTTTCT | TCAGTGACTCCTAGATGATTAA |

| | | |
|---|---|---|
| | GGGTGGGATAAGTTCTACAATGAAGT | |

Duplicate studies of the results of representative PCR experiments were shown below (**Supplemental Figure 2**).



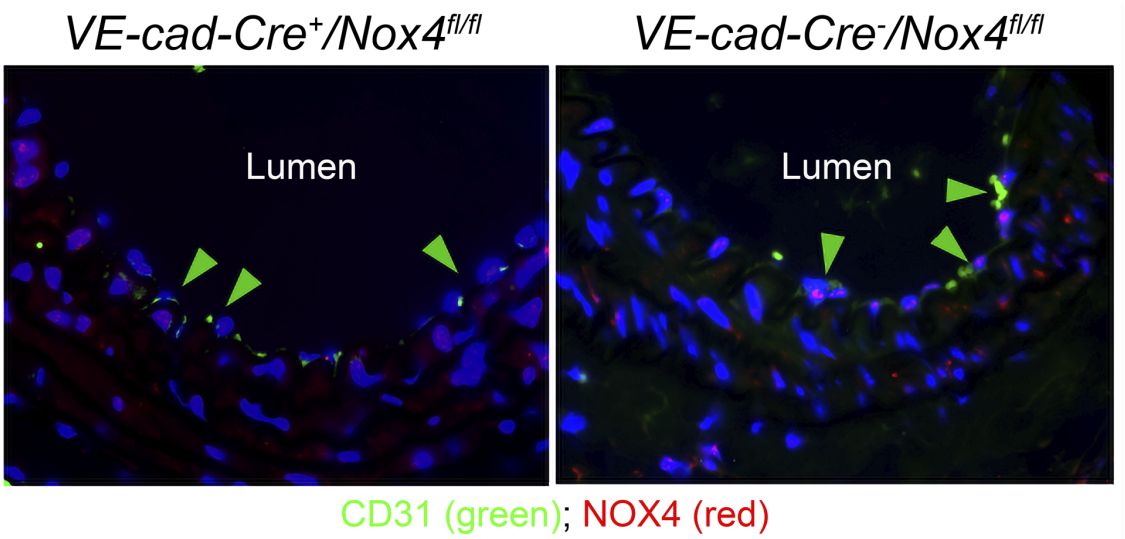

**Supplemental Figure 2. Representative real-time PCR results using primers that identify** (**A**, *VE-Cadherin-Cre* alleles; **B**, *Nox4*-flox alleles, and **C**, *Nox4*-WT alleles).

The raw data of copy number and identification of animal genotype was shown in the accompanying table (**Supplemental Table 2)**.

**Supplemental Table 2. Analysis of PCR amplicons using Cre, *Nox4*-flox, and *Nox4*-wt primers. The relative values of each probe were normalized by housekeeping gene and shown in the table.**

| **Animal** | **Cre** | ***Nox4*-flox** | ***Nox4*-wt** | **Genotype** |
|---|---|---|---|---|
| **1** | 0.002 | 0.390 | 0.931 | $Cre^{-}/Nox4^{fl/wt}$ |
| **2** | 3.998 | 0.082 | 0.566 | $Cre^{+}/Nox4^{wt/wt}$ |
| **3** | 0.007 | 0.375 | 0.001 | $Cre^{-}/Nox4^{fl/fl}$ |
| **4** | 2.640 | 0.278 | 0.000 | $Cre^{+}/Nox4^{fl/fl}$ |

Along with these confirmatory studies, additional immunofluorescence experiments demonstrated selective loss of endothelial NOX4 in the *VE-Cad-Cre*$^{+}$*/Nox4*$^{fl/fl}$ strain (**Supplemental Figure 3**).



**Supplemental Figure 3. Immunohistochemistry of aortic sections from two mice show the anticipated loss of NOX4 in endothelium in the *Nox4* KO mice (left panel) and preservation of NOX4 in mice lacking the VE-Cad-Cre recombinase (right panel)**. The endothelial cell layer was identified using antibody to CD31 (green color) and NOX4 was identified using anti-NOX4 antibody (red color). Nuclei were counterstained with DAPI (blue color).

## References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[3] J. Kuroda, T. Ago, S. Matsushima, P. Zhai, M.D. Schneider, J. Sadoshima, NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart, Proc Natl Acad Sci U S A 107(35) (2010) 15565-70.